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RESEARCH ARTICLE

Phenophysiological variation of a bee that regulates hive humidity, but not hive temperature

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ABSTRACT

Seasonal acclimatisation of thermal tolerance, evaporative water loss and metabolic rate, along with regulation of the hive environment, are key ways whereby hive-based social insects mediate climatic challenges throughout the year, but the relative importance of these traits remains poorly understood. Here, we examined seasonal variation in metabolic rate and evaporative water loss of worker bees, and seasonal variation of hive temperature and relative humidity (RH), for the stingless bee *Austroplebeia essingtoni* (Apidae: Meliponini) in arid tropical Australia. Both water loss and metabolic rate were lower in the cooler, dry winter than in the hot, wet summer at most ambient temperatures between 20°C and 45°C. Contrary to expectation, thermal tolerance thresholds were higher in the winter than in the summer. Hives were cooler in the cooler, dry winter than in the hot, wet summer, linked to an apparent lack of hive thermoregulation. The RH of hives was regulated at approximately 65% in both seasons, which is higher than unoccupied control hives in the dry season, but less than unoccupied control hives in the wet season. Although adaptations to promote water balance appear more important for survival of *A. essingtoni* than traits related to temperature regulation, their capacity for water conservation is coincident with increased thermal tolerance. For these small, eusocial stingless bees in the arid tropics, where air temperatures are relatively high and stable compared with temperate areas, regulation of hive humidity appears to be of more importance than temperature for maintaining hive health.

KEY WORDS: *Austroplebeia essingtoni*, Hymenoptera, Thermal performance, Acclimatisation, Metabolic rate, Evaporative water loss, Hive regulation

INTRODUCTION

Physiological traits provide insight into the constraints of the environment on the organism by linking organism response mechanisms to ecological patterns (Seebacher and Franklin, 2012). The two most pervasive and immediate ecophysiological constraints are energy (McNab, 2002; Tomlinson et al., 2014), largely driven by metabolic rate (Hemmingsen, 1950; Kleiber, 1961) and water, which is determined by a more complex interaction between water loss [e.g. elimination, excretion and evaporative water loss (EWL)] and water gain [e.g. drinking, preformed water and metabolic water production (MWP)] (Nagy, 2004; Woods and Smith, 2010). In both cases, the most pervasive environmental and climatic effector of animal energetics and water use is temperature (Withers, 1992).

Plasticity of physiological traits is an established component of the response of a species to environmental variability (Feder, 1987; Glanville and Seebacher, 2006; Seebacher, 2005) and falls into two categories: the acclimation of adults exposed to chronic conditions for relatively short periods (days or weeks) and seasonal acclimatisation of animals exposed to changing conditions over annual cycles (months) (Angilletta, 2009). For insects with rapid generation times, a population can acclimatise throughout a season as successive generations of adults with different ecophysiological optima emerge (Angilletta, 2009). Thermal performance curves for metabolic rates of ectotherms can be seasonally adjusted along the temperature axis, resulting in tolerance of higher temperatures in warmer seasons (Angilletta, 2009; Terblanche et al., 2010, 2005; Tomlinson et al., 2015) but also resulting in differing metabolic rates at identical temperatures across seasons (Tomlinson et al., 2015). Thermal performance curves for EWL are uncommon in the literature and acclimatisation patterns are less predictable, because both T_a and ambient relative humidity (RH) affect EWL. EWL increases as T_a increases, but decreases at high ambient RH (Withers, 1992). Although global climate patterns are complex (Peel et al., 2007), in temperate regions we would generally expect lower EWL in summer than in winter at equivalent T_a, because warmer seasons impose a more desiccating environment. However, in the arid tropics, where hot seasons coincide with high humidity and cooler seasons are drier (Peel et al., 2007), EWL acclimatisation patterns are uncertain or difficult to predict (e.g. Mogi, 2011).

Behavioural strategies can also ameliorate the challenges of environmental conditions (Casey, 1981). Most social insects regulate temperature within their nests (Fahrenholz et al., 1989; Human et al., 2006; Jones and Oldroyd, 2006; Kronenberg and Heller, 1982; Southwick and Heldmaier, 1987), with brood temperature tightly regulated within a ∼3°C range (Fahrenholz et al., 1989; Jones and Oldroyd, 2006). Regulation of hive humidity is much less studied in most social species (but see Human et al., 2006). The regulation of colony conditions in social species is often facilitated by their nest architecture (Jacklyn, 1992; Jones and Oldroyd, 2006; Sammataro and Avitabile, 2011) and also their regimented social system where different castes perform distinct duties, some of which include microclimatic regulation (Fahrenholz et al., 1989; Jones and Oldroyd, 2006).

The stingless bees (Apidae: Apinae: Meliponini) are small to medium sized (∼4 mm; Heard, 1996) eusocial bees that occur mainly in tropical and subtropical parts of the world. The Australian stingless bees (sub-tribe Trigonini; Wille, 1979) comprise the genera *Tetragonula* (Jurine, 1807) and *Austroplebeia* (Moure, 1961). *Austroplebeia* occurs across the northern parts of Australia, with a distribution congruent with the tropical and subtropical regions (Halcroft, 2012). The stingless bees have eusocial, perennial

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List of symbols and abbreviations

AH	absolute humidity
EWL	evaporative water loss
M_TR (T_{MMR})	T_a threshold where RMR or SMR of an ectotherm reaches its maximum (Tomlinson and Phillips, 2012)
MWP	metabolic water production
RH	relative humidity
RMR	resting metabolic rate
SMR	standard metabolic rate
T_a	ambient temperature
T_{hd}	T_a threshold at which RMR or SMR of an ectotherm deviates from the pure pattern of exponential increase as T_a increases (Tomlinson and Menz, 2015)
T_{ld}	T_a threshold at which RMR or SMR of an ectotherm deviates from the pure pattern of exponential increase and rapidly declines as T_a decreases (Tomlinson and Menz, 2015)
$\dot{V}_{CO_2}$	metabolic rate (rate of carbon dioxide production)
VPD	vapour pressure deficit
ΔH_c	difference in humidity between the control (uninhabited) hive and the ambient conditions
ΔH_h	difference in humidity between the inhabited hive and the ambient conditions
ΔT_c	difference in temperature between the control (uninhabited) hive
ΔT_h	difference in temperature between the inhabited hive and the ambient conditions

colonies comprising a single queen, a variable number of males (drones) and hundreds to thousands of female workers (Roubik, 1989). There is a division of labour within a hive based on the age of individual workers, where brood-rearing is undertaken by the youngest bees, that then progress as they age to nest construction and maintenance (Wille, 1983) and finally to high-risk tasks such as nest defence and foraging (Halcroft, 2012). *Austroplebeia* hives are much less structured than the hives of the honeybee (Sammataro and Avitabile, 2011), instead consisting of spherical brood, honey and pollen cells arranged into simple clusters in cavities comprising thick walls (>1.5 cm) in small trees or limbs of large trees (Halcroft, 2012; Michener, 1961; Fig. 1). Hive thermoregulation has been reported for some species of stingless bees, with bees fanning to cool hives and 'mass incubating' the brood when temperatures are low (Engels et al., 1995; Macías-Macías et al., 2011; Sakagami, 1982). The hives of *Austroplebeia australis* Friese 1898, however, generally thermoconform, with an average difference between the cavity of an artificial hive and ambient temperatures of only 0.43°C, and >2°C less than 1% of the time (Halcroft, 2012).

We studied the seasonal patterns of ecophysiological variables (phenophysiology) for the Australian stingless bee, *Austroplebeia essingtoni* Cockerell 1905, with the expectation that thermal performance of metabolic rate and EWL would differ between hot, wet summer and cool, dry winter seasons in this arid tropical environment. As for other ectotherms, the metabolic rate of *A. essingtoni* was expected to increase to a maximum T_a threshold and then rapidly decline. During the summer, we expected the upper thermal tolerance of metabolic rate (the temperature of maximal metabolic rate) to be higher than during the winter, as the bees acclimatised to warmer environmental conditions. Although we predicted EWL to increase with T_a and decrease with rising RH, the seasonal variation in RH in arid tropical Australia tends to be of much greater significance than seasonal changes in T_a, where the wet season is often nearly twice as humid as the dry but only about 10% warmer (see below). As such, we predicted that *A. essingtoni* would have lower rates of EWL in the winter, as an acclimatised response to the more desiccating climatic conditions. Since the hive is thought to be a regulated optimal microclimate for many social insects to reproduce and take refuge from unfavourable climatic conditions, we sought to link tolerance patterns of individual workers to the ecosystem by measuring seasonal patterns of hive temperature and humidity regulation. The simple nature of the hive architecture of *A. essingtoni*, however, led us to expect no regulation of the hive in correlation with physiological optima that we might identify.

MATERIALS AND METHODS

Study site

We studied three wild-caught hives of *A. essingtoni* at Broome, Western Australia (17.962°S, 122.236°E), which has a semi-arid tropical climate with two distinct seasons. The dry season extends from April to November, with an average maximum temperature of 29.5°C, average rainfall of 9.3 mm and RH 34.6% at 15:00 h in June–August (winter) (Australian Bureau of Meteorology). The wet season from December to March is characterised by comparatively hot and rainy conditions of 33.5°C, 139.4 mm average rainfall and RH 64.3% between December and February (summer) (Australian Bureau of Meteorology). The Dampier Peninsula, where Broome is situated, represents the southernmost limit on the west coast of Australia for *A. essingtoni*, which appears to be widespread across the dry tropics of northern Australia (Atlas of Living Australia).

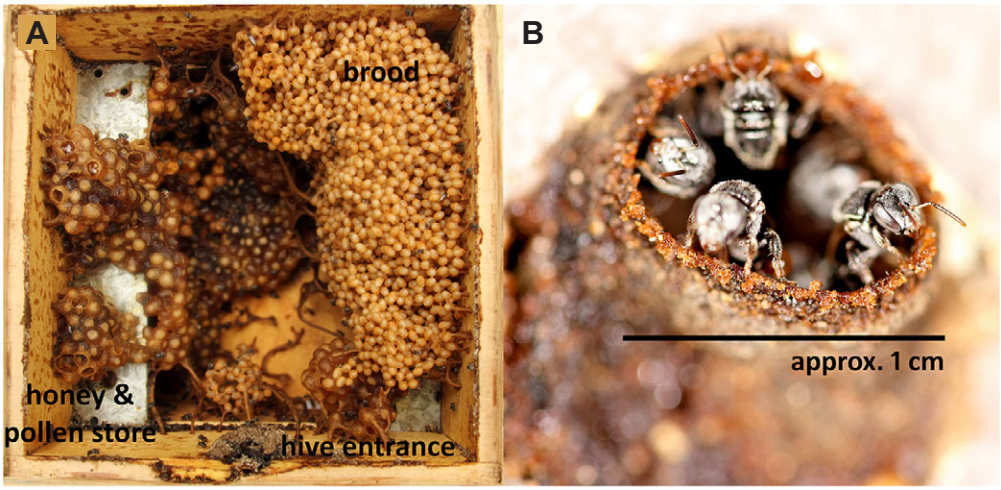

Fig. 1. A hive of *Austroplebeia essingtoni* bees. (A) The internal arrangement of a hive of *A. essingtoni*, showing only partial segregation of components of the hive, similar to the hive organisation of *A. australis* (Halcroft, 2012). There are irregular clusters of brood cells, honey pots and pollen pots, although honey and pollen are maintained in a separate area of the hive to brood. (B) A cluster of *A. essingtoni* inside the entrance tube to a hive, illustrating the small size of the entrance and the bees.

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Our three captive hives were translocated to Broome from neighbouring private properties approximately 24 months prior to the study (T. Heard, Sugarbag). They were housed in artificial hives ~20×20×18 cm in size constructed of 4-cm-thick pinewood. Hive condition was assessed by weighing the hives regularly, as suggested for apicultural hives of *Apis mellifera* (Warré, 1948). Throughout the 2013–2014 year of this study, the mass of the hives did not fluctuate seasonally, averaging ~3400 g (Fig. S1). The hives were exposed to natural daily and seasonal fluctuations in climate and photoperiod. During the hot, wet summer the bees were active from sunrise to sunset, and did not appear to avoid any climatic conditions except for rainfall, but during the cooler, dry winter they were inactive until midmorning and appeared to avoid temperatures lower than approximately 20°C (Ayton, 2014). During summer (February 2014), we collected 16 worker bees from each hive as they were leaving on foraging flights, totalling 48 bees. In the dry winter (June 2014) one of the hives had declined in size to such an extent that it was excluded from the study so as not to threaten its survival. We collected 25 bees from each remaining hive, totalling 50 bees.

Respirometry

Naive bees were collected leaving the hives and transported to the laboratory in vials immediately prior to respirometry trials and cooled in a refrigerator to ensure their compliance during handling. Each individual bee was weighed (while quiescent) before and after metabolism trials and allowed to warm up to room temperature (~25°C) for 30 min prior to experimental treatment. Metabolic rate (rate of CO_2 production, $\dot{V}_{CO_2}$) and EWL were measured using a flow-through respirometer (Withers, 2001). The incurrent air stream was scrubbed of water using Drierite desiccant (W. H. Hammond Drierite Company) and carbon dioxide using soda lime (Sigma), and passed through the respirometry chamber at a flow rate of 105.6 ml min^{-1} (STPD), controlled using the internal flow controller of a Licor 6400XT analyser/pump system. Partial pressures of CO_2 and H_2O vapour were measured by infra-red gas analysis (IRGA) and logged by the Licor every 10 s using the Licor 'insect respiration' algorithm. Water vapour was not removed prior to CO_2 analysis, but the Licor 6400 algorithms compensate for this. The LiCor 6400 system has a series of factory calibrations constructed around a third-order polynomial function that accounts for water vapour pressure (0–100% RH) and a fifth-order polynomial that accounts for CO_2 concentrations (0–3000 µmol mol^{-1}), standardised across a temperature range of 15–45°C. Licor discourage recalibration of the units because of the complexity and sensitivity of their factory calibration process and we used the factory settings during our measurements. Baseline readings of background CO_2 were established for at least 10 min before and after the measurement trials where individual bees were exposed to one of the experimental temperatures for 30 min. A range of temperature (T_a) treatments was tested, spanning those naturally experienced during the daytime activity period in Broome, from 20°C to 45°C. The T_a treatments were 20, 25, 35 and 40°C during the summer and 20, 25, 35, 40 and 45°C during the winter. Each individual bee was exposed to only a single, acute, 30 min temperature treatment and then kept as a specimen. Counterintuitively given the cooler climatic conditions, we had to make measurements at higher T_a treatments during the winter than in the summer in order to gather data on the upper tolerance limits of the bees. The experimental temperature treatments were controlled using a custom-built incubator.

Data were analysed using a custom-written VisualBasic (Microsoft) program to calculate the average $\dot{V}_{CO_2}$ and EWL for the lowest and most stable 20 min period of each respirometry trial (Withers, 2001). When checked at 30 min intervals at the end of the trials, *A. essingtoni* were always quiescent at each temperature. The digestive state of the bees was unknown since they were harvested when exiting the hives throughout the day. As such, metabolic rates measured in this study probably represent resting metabolic rate (RMR), rather than standard metabolic rate (SMR) (IUPS Thermal Commission, 2003).

Hive temperature and humidity

To examine regulation of hive temperature and humidity, each of our three experimental hives was paired with a nearby empty hive (within 1 m distance) of identical construction and colour and all hives were fitted with a temperature/RH data logger (HOBO H21-002, Onset Computer Corporation). The probes were inserted through a hole drilled into the hive space, opposite the entrance used by the bees, and wrapped in gauze to prevent the bees covering the probe with propolis (rendering the RH measurements inaccurate). A temperature/RH probe was fitted to the shaded underside of the hives to record local ambient T_a and RH. All three associated T_a/RH probes for each hive (inhabited hive, control hive and ambient) were monitored by a single HOBO H21-002 data logger; temperature was measured to an accuracy of ±0.7°C (precision=0.02°C) and relative humidity (RH) to an accuracy of ±5% (precision=0.1%) every 5 min for a year from February 2014 to February 2015.

For the purposes of testing seasonal patterns, we analysed a 4 week subset of the data collected in each season (June and January), coinciding with our metabolic studies. We calculated absolute humidity (mg cm^{-3}) from T_a (°C) and RH (%) using hygrometric equations (Parrish and Putnam, 1977). We also calculated the differential for the occupied and control hives from ambient conditions for temperature (ΔT_h and ΔT_c, respectively) and for absolute humidity (ΔH_h and ΔH_c, respectively) by subtracting the corresponding ambient value from that recorded inside the hive or control box. The relative humidity and temperature data were used to quantify the desiccating quality of the environment in terms of the vapour pressure deficit (VPD) by calculating the saturation vapour pressure at each ambient temperature (Murray, 1967) and comparing this with the vapour pressure for the measured RH (Monteith and Unsworth, 2007).

Statistical analysis

Variation in the body mass of individual bees was tested using a linear model (LM) of measurement season, hive of origin and experimental T_a, where T_a was treated as a categorical variable. A model-averaging approach using the 'MuMIn' package for R 3.0.3 (https://cran.r-project.org/package=MuMIn) provided the most parsimonious model of variation in body mass [using Akaike information criterion values (AICc)] (Burnham and Anderson, 2002). The most parsimonious model was subsequently interrogated by ANOVA to interpret the relative significance of the contributory factors. The effects of respirometry exposure on body mass were tested by calculating the loss in body mass for each respirometry trial and fitting an exponential regression between mass loss and experimental temperature. This regression was then grouped by season and compared with the consensus model by AIC. All $\dot{V}_{CO_2}$ measurements were allometrically corrected using $mass^{0.75}$ (Chown et al., 2007) and EWL measurements were allometrically corrected using $mass^{0.67}$ (Chown et al., 1998; Edney, 1977) prior to statistical analysis.

The response of metabolic rate to temperature should conform to a unimodal, non-linear function (Angilletta, 2006; Tomlinson et al.,

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2015; Tomlinson and Phillips, 2015). We fitted a three-part (tri-exponential) function (Tomlinson and Menz, 2015) of the form:

$$\dot{V}_{CO_2} = y_0\ (e^{kT_a} - e^{l-T_a} - e^{T_a-h}), \quad (1)$$

where y_0 is the intercept of the curve at 0°C, k estimates the exponential rate of change of $\dot{V}_{CO_2}$ with T_a, and l and h represent lower and upper thermal constraints, respectively. The most parsimonious model permutation was then assessed by AIC using the 'MuMIn' package for R, and used to interpret patterns of thermal tolerance. The temperature of peak metabolic response M_TR (Tomlinson and Phillips, 2012, 2015) or T_{MMR} (Tomlinson et al., 2015; Tomlinson and Menz, 2015), was calculated as the first-order derivative using the numDeriv package (https://cran.r-project.org/package=numDeriv), and solving using the 'uniroot' function (Brent, 1973). Two points of deviation from the base exponential increase of $\dot{V}_{CO_2}$ with T_a were estimated by solving the second-order derivative. This gave T_{ld}, the lower point of deviation where $\dot{V}_{CO_2}$ declines rapidly in response to chill and T_{hd}, the upper point of deviation, analogous to T_d (Tomlinson and Phillips, 2015), where $\dot{V}_{CO_2}$ departs from a simple exponential increase in response to accumulating heat stress.

We used non-linear least-squares regression (NLS) to model the exponential influence of effect T_a on EWL (Tomlinson et al., 2015; Tomlinson and Menz, 2015; Tomlinson and Phillips, 2015):

$$\mathrm{EWL} = y_0 e^{kT_a}, \quad (2)$$

where y_0 is the intercept of the curve at 0°C and k estimates the exponential rate of change of EWL with T_a.

In the case of all non-linear regression models, unique permutations of each function were parameterised by partitioning the data with season as a co-factor (Table 1) to yield functions combining parameters unique to each season, and common across both seasons. The most parsimonious permutation of the model was then assessed by AIC using 'MuMIn'.

To test for effects of season, hive identity (i.e. hives one, two and three) and box type (i.e. inhabited, control and ambient) on temperature and humidity measurements, we used a linear model (LM), and applied model averaging using 'MuMIn'. To interpret the effects of differences between seasons and box type, we fitted linear models to the ΔT_{hive}, $\Delta T_{control}$, ΔH_{hive}, $\Delta H_{control}$, ΔVPD_{hive} and $\Delta VPD_{control}$ values with respect to the associated ambient conditions (T_a, H_a and VPD_a) where ambient conditions were nested within each unique season×box combination (e.g. inhabited×dry winter, inhabited×wet summer, control×dry winter and control×wet summer). The most parsimonious models were examined using ANOVA to test the significance of each parameter. Statistical analyses were performed in R version 3.0.3 (https://cran.r-project.org). Data are presented as means±s.e.

RESULTS

Differences in body mass

Hive identity and season were present in all four of the top models for body mass, the others of which also included experimental temperature and various interaction effects (Table 1). The most parsimonious model of body mass was characterised by only hive identity ($F_{2,89}$=26.5; P=9.37×10^{-10}) and season ($F_{1,89}$=7.21; P=0.00866). The species average mass was 4.6±0.08 mg, (4.7±0.08 in winter, 4.5±0.15 in summer). Hive 1 had the heaviest individuals (4.9±0.11 mg), followed by hive 3 (4.6±0.13 mg) and hive 2 (3.9±0.14 mg). The most parsimonious linear model of mass loss during respirometry trials (AICc=156; ΔAIC=0.00) resolved a higher constant (intercept) for body mass loss during respirometry in the wet summer (0.606±0.216) than in the dry winter (0.163±0.079), although the effect of temperature (slope) was not different between seasons (0.020±0.011). The proportion of body mass lost during respirometry trials in the hot, wet season was 27.5±3.28% (ranging from 19.2±9.8% at 15°C to 39.7±7.7% at 40°C), whereas the proportion of mass loss in the cooler, dry season was 6.9±0.87% (ranging from 4.5±1.8% at 20°C to 15.7±4.3% at 45°C).

Table 1. Information criterion comparisons of models for body mass, metabolic rate and evaporative water loss

	AICc	Δ AIC
mass=hive+season	−1125.9	0.00
mass=[T_a+hive+season+T_a] ×season	−1125.3	0.62
mass=[T_a+hive+season+T_a] ×[hive+T_a]×season	−1124.3	0.88
mass=[T_a+hive+season+T_a] ×[hive+T_a]×[season+hive] ×seasonT_a×hive×season	−1124.3	1.65
$\dot{V}_{CO_2} = y_0$[season] ×($e^{k\,T_a} - e^{T_a-h[\mathrm{season}]} - e^{l[\mathrm{season}]-T_a}$)	150.38	0.00
$\dot{V}_{CO_2} = y_0 \times (e^{k[\mathrm{season}]} \times T_a - e^{T_a-h[\mathrm{season}]} - e^{l[\mathrm{season}]-T_a})$	150.89	0.50
$\dot{V}_{CO_2} = y_0$[season] ×($e^{k[\mathrm{season}]\times T_a} - e^{T_a-h[\mathrm{season}]} - e^{l[\mathrm{season}]-T_a}$)	152.80	2.42
$\dot{V}_{CO_2} = y_0 \times (e^{k\times T_a} - e^{T_a-h[\mathrm{season}]} - e^{l[\mathrm{season}]-T_a})$	155.00	4.62
EWL = $y_0 \times (e^{k[\mathrm{season}]\times T_a})$	−245.03	0.00
EWL = y_0[season] × ($e^{k\times T_a}$)	−242.90	2.13
EWL = y_0[season] × ($e^{k[\mathrm{season}]\times T_a}$)	−242.76	2.27
EWL = y_0($e^{k\times T_a}$)	148.90	96.13

Information criterion comparisons of models for body mass show the four best generalised linear models of patterns influencing body mass (hive indicates unique hive identities); patterns influencing metabolic rate and evaporative water loss were best described by non-linear models, where y_0=the projected intercept of the exponential base function at T_a=0°C, k=the exponential scaling exponent, h=the upper thermal limit of the function, and l=the lower thermal limit of the function. Each function describes a unique permutation of the thermal performance model where [season] denotes the estimation of unique coefficients for each season. Coefficients are otherwise common for both seasons. Note that, for metabolic rate it was not possible to resolve common parameters for h or l. The model describing the best fit is denoted with ΔAIC of 0.00, and increasing ΔAIC are associated with lesser fit. Models within ΔAIC of 2.00 are generally considered equally parsimonious.

Thermal performance of metabolic rate

Untransformed data can be found in Fig. S1 and Table S1. The thermal performance curve for allometrically corrected metabolic rate (Fig. 2) showed the expected, tri-exponential curve (Tomlinson and Menz, 2015). It was not possible to fit a single convergent performance function to the complete data set, but curves with an array of unique parameter estimates of thermal tolerance thresholds could be fitted to each season (Table 1). The maximum temperature threshold of each model was higher in the dry winter (M_TR=43.9±0.08°C, T_{hd}=40.9±0.05°C, T_{ld}=25.2±0.05°C) than in the wet summer (M_TR=37.2±0.05°C, T_{hd}=36.4±0.05°C, T_{ld}=17.1±0.05°C). The most parsimonious function also found unique seasonal coefficients for y_0 (Table 1), but an equally plausible model which estimated common y_0 and seasonally unique k (ΔAIC=0.50, Table 1).

Thermal performance of evaporative water loss

Untransformed data can be found in Fig. S1 and Table S1. Allometrically corrected EWL increased exponentially with

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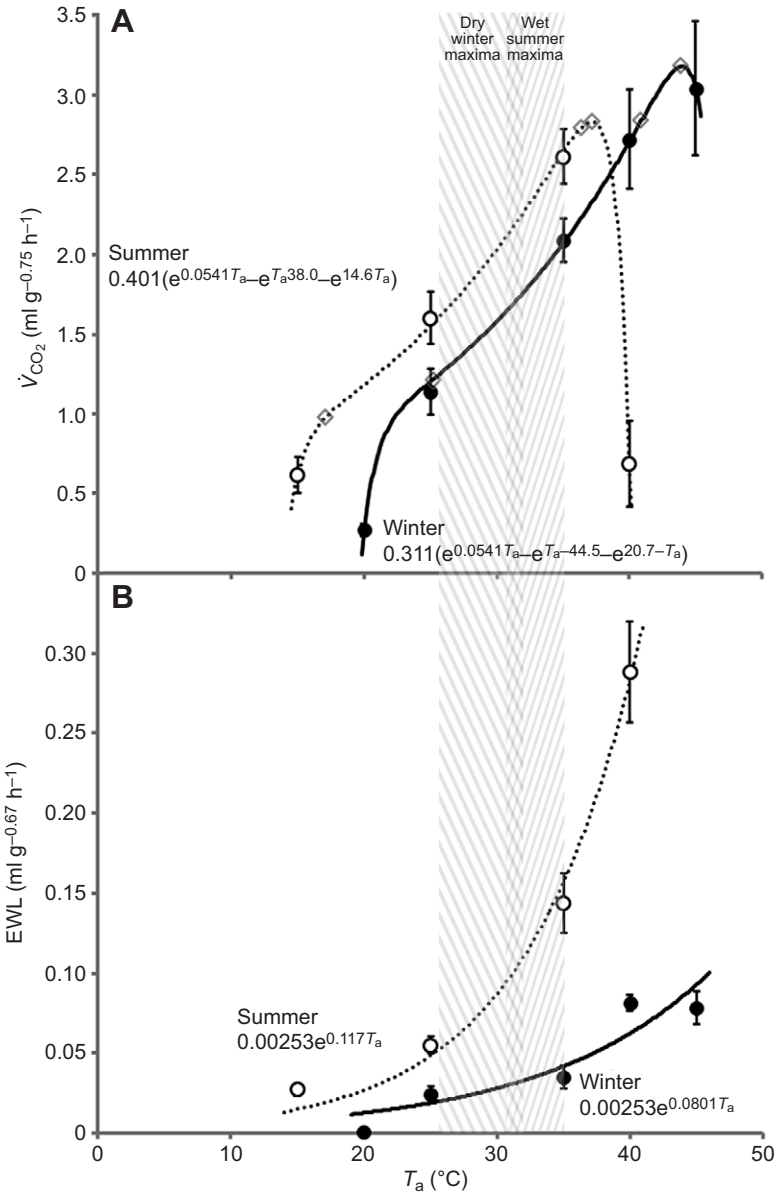

Fig. 2. Comparison of thermal performance curves of *Austroplebeia essingtoni*. (A) Metabolic rate, where thermal tolerance thresholds and M_TR all occurred at higher T_a in the cool, dry winter (filled circles and solid line) than in the hot, wet summer (open circles and dashed line). Critical thermal thresholds (diamonds) are indicated for each season. (B) Evaporative water loss, where water loss increased more rapidly with increasing T_a in the summer (open circles) than in the winter (closed circles). Black lines indicate non-linear fits for the winter, dashed lines indicate nonlinear fits for the summer. Cross-hatched regions represent the 10th–90th percentile maximum temperatures for Broome in February (summer) and July (winter) recorded by the Australian Bureau of Meteorology. Data are means±1 s.e.m., n=10, N=40 for summer; n=10, N=50 for winter.

increasing temperature (Fig. 2). The most parsimonious model found unique temperature scaling coefficients (k) for each season and unique seasonal minimal EWL (y_0; Table 1). EWL was substantially higher in the hot, wet summer than in the cooler, dry winter at all T_a.

Seasonal patterns of hive conditions

There was a peak in the temperature of inhabited hives at 13:00 h during the cooler, dry winter, and a minimum at 06:00 h (Fig. 3). During the hot, wet summer, the peak occurred at 11:00 h and lasted longer, whereas the minimum occurred at 02:00 h. Similar diurnal patterns were also evident for the uninhabited hive and ambient conditions. During daylight hours, the dry winter was cooler (T_a=23.2±0.06°C; T_{hive}=22.8±0.05°C) than the wet summer (T_a=26.9±0.04°C; T_{hive}=26.9±0.04°C). There were effects of hive identity ($F_{2,37404}$=12,148; P=2.20×10^{-16}), season ($F_{1,37404}$=26,828; P=2.20×10^{-16}) and hour ($F_{1,37404}$=1805; P=2.20×10^{-16}) on hive temperature in the most parsimonious model of hive conditions. The most parsimonious model of hive temperature also included significant influences of the interaction terms hive:hour (P=2.20×10^{-16}), hive:season (P=2.20×10^{-16}), hour:season (P=2.20×10^{-16}) and hive:hour:season (P=4.18×10^{-14}).

There were significant linear relationships between the ΔT of the hive and T_a ($F_{1,74824}$=186; P=2.00×10^{-16}), which differed between seasons ($F_{1,74824}$=3214; P=2.00×10^{-16}) and between inhabited and control hives ($F_{1,74824}$=1343; P=2.00×10^{-16}; Fig. 4). All the potential interaction terms were also significant ($P_{Ta:season}$=2.00×10^{-16}; $P_{Ta:box}$=1.03×10^{-4}; $P_{Ta:season}$=2.00×10^{-16}; $P_{season:box}$=2.00×10^{-16}; $P_{Ta:season:box}$=2.00×10^{-16}). In both seasons, the slope of the relationship was nearly zero, indicative of very similar temperatures inside and outside both the unoccupied and the occupied hives. Although there were statistical differences between control and inhabited hives, the magnitude of the difference was very small (summer minimum ΔT_h=0.08±0.01°C, summer maximum ΔT_h=0.24±0.02°C, winter minimum ΔT_h=0.15±0.0°C, winter maximum ΔT_h=0.40±0.03°C; Fig. 3).

RH reached its minimum in the inhabited hives at 24:00 h during the dry winter, and peaked at 17:00 h (Fig. 3). During the wet summer, the minimum RH occurred at 17:00 h, while the peak occurred at 06:00 h. These patterns were distinctly different to

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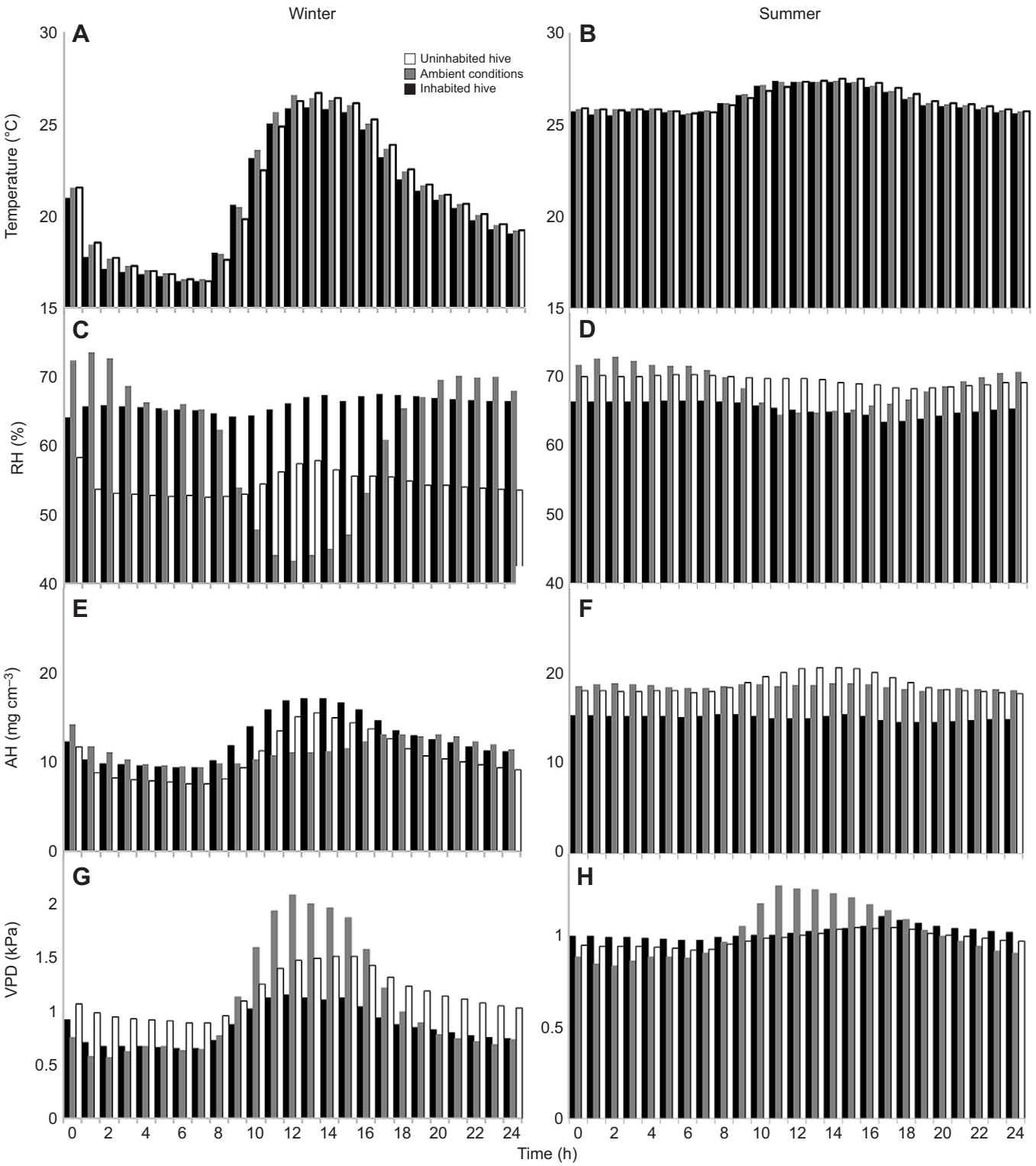

Fig. 3. Climate inside the hives of *Austroplebeia essingtoni* relative to ambient conditions and unoccupied control hives. Average temperature (A,B), relative humidity (C,D), average absolute humidity (E,F) and average vapour pressure deficit (G,H) at each hour in the cooler, dry winter (A,C,E,G) and the hot, wet summer (B,D,F,H). Data are means over a 28 day period (n=24, N=192 for winter; n=13, N=224 for summer); s.e.m. are omitted for clarity.

ambient conditions, where minimum RH occurred near midday, and conditions were much more variable than inside the hives. During daylight, the dry winter was less humid than in the wet summer outside the hives (winter ambient RH=52.5±0.25%; summer ambient RH=66.7±0.08%), but not inside the hives (winter hive RH=66.0±0.07°C; summer hive RH=65.3±0.08%). Similarly to hive temperature, hive humidity was related to hive identity ($F_{2,29342}$=2249; P=2.20×10^{-16}), season ($F_{1,29342}$=1576; P=2.20×10^{-16}) and hour of day ($F_{1,29342}$=25.6; P=4.15×10^{-7}) and the most parsimonious model also included interactions of hive:hour (P=4.72×10^{-16}), hive:season (P=2.20×10^{-16}), hour:season (P=2.20×10^{-16}) and hive:hour:season (P=6.31×10^{-4}). The most

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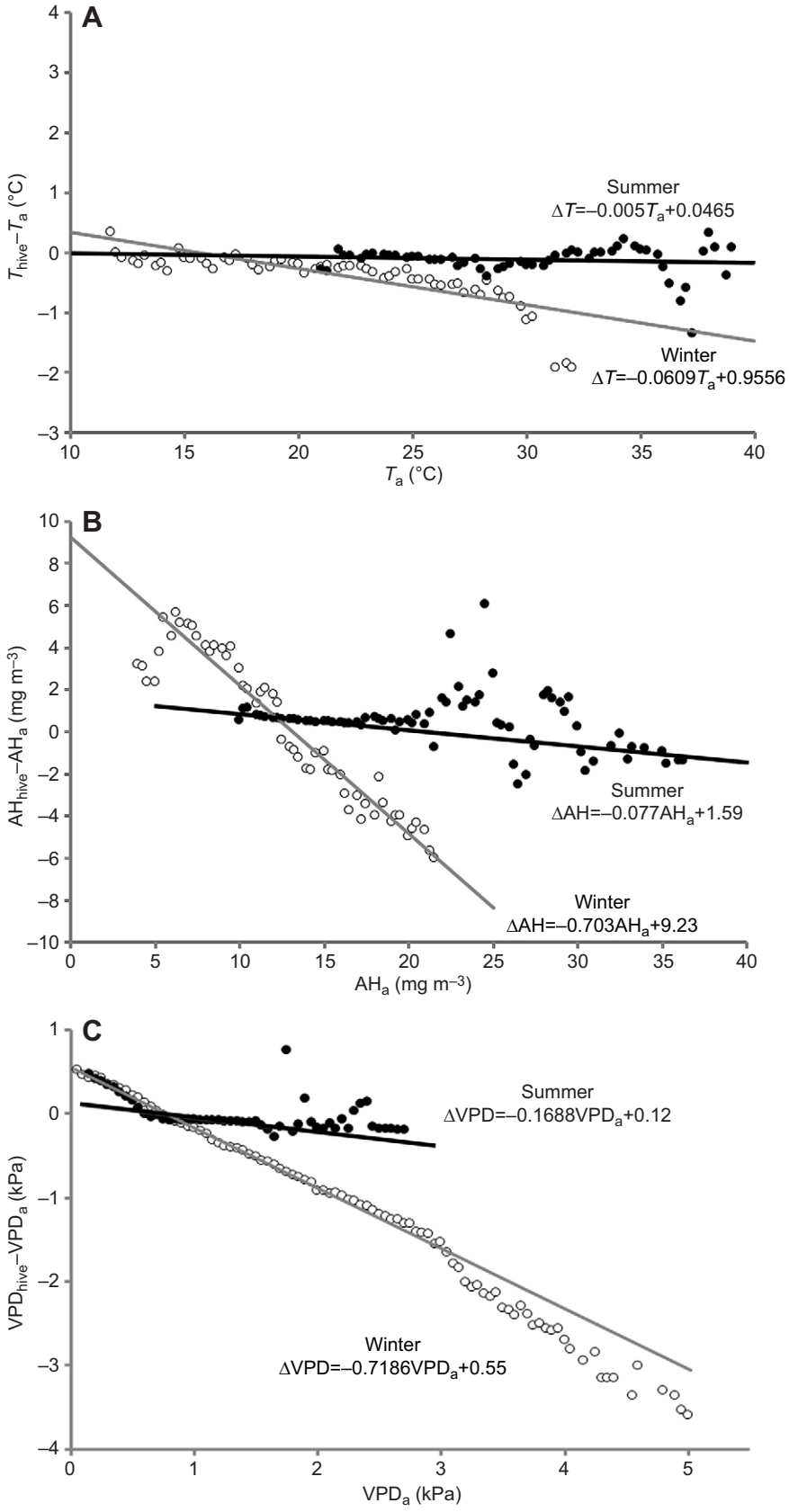

Fig. 4. The mean differentials between the conditions of inhabited hives and ambient conditions for each season in *Austroplebeia essingtoni*. (A) Temperature is not obviously different inside the hive compared with ambient conditions, where the intercept and slopes of the lines are both nearly zero, in all hive boxes, for both seasons. (B) Humidity also appears relatively similar to ambient conditions inside the hives in the hot, wet summer, but in the cooler, dry winter the hives are drier at high ambient humidity and moister at low ambient humidity in both inhabited and uninhabited boxes. (C) When temperature and humidity are combined in the form of the vapour pressure deficit, the VPD inside the inhabited hives is almost equivalent to that outside the hives in the summer, but in the winter it is heavily modified (n=24 and N=192 for winter; n=13, N=224 for summer).

desiccating conditions (highest VPD) were reached between 11:00 h and 14:00 h in the dry winter in both uninhabited and inhabited boxes, and were at their minimum between 01:00 h and 04:00 h (Fig. 3). During the wet summer, the highest VPD occurred between 16:00 h and 18:00 h, minimally desiccating conditions extended for a much greater time, between 20:00 h and 12:00 h, and

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VPD patterns were much more uniform throughout the day. Hive VPD was related to hive identity ($F_{2,29342}$=1676; P=2.20×10^{-16}), season ($F_{1,29342}$=4319; P=2.20×10^{-16}) and hour of day ($F_{1,29342}$=745; P=2.20×10^{-16}), and the most parsimonious model also included interactions of hive:hour (P=2.22×10^{-14}), hive:season (P=2.20×10^{-16}), hour:season (P=2.16×10^{-11}) and hive:hour:season (P=0.00808).

There were significant linear relationships between the ΔH of the hive and ambient humidity ($F_{1,66760}$=3595; P=2.00×10^{-16}), which differed between seasons ($F_{1,66760}$=1484; P=2.00×10^{-16}) and inhabited and control hives ($F_{1,66760}$=732; P=2.00×10^{-16}; Fig. 4). All the potential interaction terms were also significant ($P_{AH:season}$=2.00×10^{-16}; $P_{AH:box}$=1.03×10^{-4}; $P_{AH:season}$=2.00×10^{-16}; $P_{season:box}$=2.00×10^{-16}; $P_{AH:season:box}$=2.00×10^{-16}). In both seasons, the humidity inside the inhabited hives deviated markedly from the ambient conditions, maintaining higher humidity in dry conditions, and lower humidity in humid conditions. In the wet summer this effect was substantially less powerful than in the dry winter, as indicated by the shallower slope of the linear fit ($m_{wet\ hive}$=−0.077, $m_{dry\ hive}$=−0.703). There was little difference between ambient and control hives in the wet summer ($m_{wet\ control}$=0.053), but a substantial decrease in the difference between ambient conditions and the control hives in the dry winter ($m_{dry\ control}$=−0.838; Fig. 4). In the dry winter, the average humidity of the inhabited hives (min=64.1±0.16%, 12.2±0.11 mgH_2O cm^{-3}; max=67.4±0.22%, 14.5±0.09 mgH_2O cm^{-3}) was higher than that of the uninhabited hives (min=52.5±0.43%, 7.5±0.10 mgH_2O cm^{-3}; max=58.2±0.34%, 11.7±0.17 mgH_2O cm^{-3}) by ∼12%. Conversely, in the wet summer, the inhabited hives were on average approximately 2% less humid (min=64.1±0.16%, 12.2±0.11 mgH_2O cm^{-3}; max=66.6±0.29%, 15.1±0.11 mgH_2O cm^{-3}) than the uninhabited hives (min=68.3±0.36%, 18.9±0.23 mgH_2O cm^{-3}; max=70.3±0.34%, 17.9±0.17 mgH_2O cm^{-3}; Fig. 4). While a hive insulated the internal environment against the daily variation in relative humidity, the presence of the bees also raised the relative humidity in the dry season and reduced it in the wet season. There were significant linear relationships between the ΔVPD of the hive and ambient VPD ($F_{1,74824}$=1.01×10^{33}; P=2.00×10^{-16}), which differed only on the basis of the VPD×season×season:box interaction (P=0.0207). In the wet season, the VPD inside the hive was similar to ambient conditions, but in the dry season the internal environment was comparatively less desiccating as the external VPD became more desiccating. Overall the pattern of RH and VPD were congruent, since, in the absence of controlled temperatures, lower RH in the hive naturally relates to higher VPD relative to the control and vice versa.

DISCUSSION

We found strong evidence for seasonal acclimatisation of metabolic rate, thermal tolerance and EWL for *A. essingtoni*. Whereas we predicted a higher thermal tolerance in the hot, wet summer for metabolism, as measured by the point where $\dot{V}_{CO_2}$ begins to decline with increasing temperature, we actually found higher thermal tolerance associated with the cooler, dry winter. The seasonal acclimatisation of EWL resulted in higher desiccation resistance associated with winter conditions. This is contrary to expectations that in cooler conditions there would be less selection pressure to reduce water loss, but congruent with expectations that EWL should be reduced by a small insect in a more desiccating climate. Given that lower temperatures are associated with arid, desiccating conditions in our study region, it appears that thermal tolerance is of less critical importance to *A. essingtoni* than resistance to desiccation. This is also consistent with their small size and the moderate environmental T_a, which suggest high rates of EWL, but lesser challenges of high temperature, because heat is readily lost by small ectotherms. The fact that EWL shows seasonal patterns consistent with our predictions, increasing desiccation resistance in the more arid season, but thermal tolerance thresholds increase in the cooler season, far beyond their climatic relevance, suggests that patterns of thermal tolerance might be inextricably linked to patterns of EWL. We conclude from the following discussion that *A. essingtoni* appear not to regulate hive temperature, but unexpectedly, we found that they do regulate hive humidity, and are more rigorous in this regulation in the cooler dry winter than in the warmer wet summer.

Metabolic rate

There are two parameters in exponentially based thermal performance curves (e.g. Tomlinson et al., 2015; Tomlinson and Menz, 2015; Tomlinson and Phillips, 2015) that offer insight into thermal effects on metabolic rate. The thermal scaling exponent k is analogous to the slope of a linear regression, indicating how quickly $\dot{V}_{CO_2}$ increases per degree increase in T_a. The intercept y_0 is indicative of the minimum maintenance requirement of the organism. The common k between T_a and $\dot{V}_{CO_2}$ for both seasons suggests that the bees were not acclimatising to temperature directly. Rather, they had a higher asymptotic $\dot{V}_{CO_2}$ (y_0) in the wet summer than in the dry winter. Hence, acclimatisation may be acting to match metabolic rates with ecological energy availability in the wet summer, which is the peak season for flowering and nectar production in tropical Australia (Boulter et al., 2006). High metabolic rate is correlated with the maintenance of highly active flight muscles (Suarez, 2000; Suarez et al., 1996), and higher activity. Therefore, higher resting metabolic rates in the wet summer may enhance the activity and foraging efficiency of *A. essingtoni* during the period of peak food availability.

The results of the respirometry trials do not support the hypothesis that *A. essingtoni* have a higher upper thermal tolerance in the summer than in the winter. We predicted that the wet summer would be more thermally challenging to *A. essingtoni* than the dry winter. In summer, the point of peak metabolic activity (M_TR) was similar to average diurnal environmental temperatures, but much higher than the environmental temperatures in the dry winter (Fig. 2). However, counter to our expectations, the upper thermal tolerance thresholds were higher in the cooler dry winter than the hot wet summer, and far exceeded the climatic conditions that they encountered. This contrasts with studies of other insect taxa, where increased thermal tolerance is associated with seasonal increases in climatic temperature (Berrigan, 1997; Terblanche et al., 2006; Tomlinson et al., 2015). Our data for *A. essingtoni*, however, parallel laboratory findings for *Drosophila buzzatii* (Sørensen et al., 2001; Sørensen and Loeschcke, 2002), where short day lengths were artificially associated with warmer conditions in a way that ran counter to the expected order. Under these conditions, higher thermal tolerance was associated with long day length, regardless of climatic temperatures. While photoperiod is not likely to be the driving factor for thermal tolerance in our natural system, since long photoperiod remained linked with high ambient temperatures in the summer, it does demonstrate that thermal tolerance patterns can be driven by factors other than temperature. In the case of *A. essingtoni*, given the high rates of water loss during the respiratory trials, we suggest that lower thermal tolerance in the dry season may be driven by increased EWL.

Although the seasonal patterns that we observed for metabolic rate could represent an acclimatisation to changing seasonal conditions, it is likely to be a developmental acclimatisation pattern resulting from the emergence of new generations of brood

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over the 6 months between measurement periods (Angilletta, 2009). While some species of *Austroplebeia* have average lifespans of ~160 days in subtropical climates (Halcroft, 2012) and hence a small number of the same adults could have been alive in both our measurement seasons, this is unlikely in free-living bees subject to natural predation, disease and other ecological processes. Developmental acclimatisation results from an accumulation of changes in cell structure and biochemistry that enhances endurance to environmental strain caused by particularly challenging climatic factors (Bowler, 2005), resulting in adults that are better adapted to the climatic conditions in which they emerge (Angilletta, 2009).

Evaporative water loss

The seasonal acclimatisation of lower EWL in winter is consistent with our predictions based on climatic patterns in the arid tropics. The lower ambient RH (and increased ambient VPD) of the dry winter represents a more challenging environment for water balance of bees than the wet summer. Animals evaporate water to the atmosphere at less than about 99.4% RH (Willmer et al., 2009), and the rate of loss increases according to the RH differential between the body (assumed to be 100%) and the atmosphere. In the summer, the average diurnal environmental RH was nearly 67%, but in the dry winter it decreased to 52%, which indicates an increased water vapour pressure deficit. In the winter, the bees lose less water by evaporation as temperature increases (the scaling exponent k is lower) compared with the summer. Therefore, based on the thermal performance curves (Fig. 2), the bees would desiccate more slowly in the dry winter than in the wet summer, even at temperatures that far exceed the climatic conditions that free-ranging bees would encounter in this season.

Our data suggest that reduced ambient RH, hence a more desiccating climate, rather than T_a drives seasonal acclimatisation of *A. essingtoni* in the form of increased desiccation resistance. Since temperature is also typically a powerful climatic forcing factor often associated with lower RH and thus influencing EWL (Cloudsley-Thompson, 1991; Withers, 1992), it is intuitive to expect greater desiccation resistance in hotter seasons. However, in many tropical systems where high humidity occurs in hot seasons, greater resistance to desiccation at low RH may coincide with cooler ambient conditions, as in our data (Fig. 2).

Hive regulation

While we found that inhabited hives were statistically cooler than uninhabited hives, the slope of the association between temperature differentials and ambient conditions was consistently close to zero. The magnitude of the differences in temperature between inhabited hives and ambient conditions was consistently less than 1°C – a difference sufficiently small as to have limited biological impact – despite its statistical significance (Anderson et al., 2001; Johnson, 1999). Furthermore, the T_a regressions of control boxes and inhabited hives were so similar as to be nearly indistinguishable (Fig. 4), reinforcing our scepticism of the biological value of this effect. We instead conclude that, as hypothesised, *A. essingtoni* do not actively thermoregulate the hive. Our finding is consistent with the thermoconformity reported for *A. australis*, although Halcroft (2012), using much more localised sensors found that *A. australis* could raise the temperature of their brood. It is possible that *A. essingtoni* may similarly manage T_a for the brood, but not the entire hive.

Our results for hive temperature are strikingly different to a similar study of the western honeybee *Apis mellifera*, in a temperate (hot and dry summer, cool and wet winter) system (Human et al., 2006), which showed substantial thermoregulation of the hive in comparison with both ambient conditions and uninhabited control hives. Hive thermoregulation in response to experimental heating and cooling has also been demonstrated for some species of stingless bees outside Australia, including *Tetragonula spinipes* and *Scaptotrigona postica* (Engels et al., 1995; Sakagami, 1982) from the wet tropics, where the brood was maintained between 29 and 39°C, regardless of ambient conditions ranging from 25 to 45°C (Engels et al., 1995). Engels et al. (1995) did note, however, that thermoregulation efforts were abandoned relatively rapidly in the face of overheating, and that *S. postica* did not gather water to facilitate evaporative cooling of the hive, as honey bees do (Human et al., 2006; Lindauer, 1955b). A capacity to thermoregulate the hive has also been speculated for the wet-tropical *Melipona colimana* (Macías-Macías et al., 2011). However, these species tend to occur in a broader range of climates than the strictly arid-tropical *A. essingtoni*. Hence, the more perennially amenable conditions of the tropics may have relaxed selection pressure for hive thermoregulation by *A. essingtoni*, whereas this may be a necessity for colony survival in thermally variable environments.

What was a more surprising outcome of our study of hive conditions was the ability of *A. essingtoni* to regulate hive humidity to a consistent level that, in the dry winter, involved raising the internal RH of the hive, but that, in the wet summer, involved reducing the internal RH (Fig. 3). In terms of the vapour pressure deficit (VPD), the inhabited hives present a less desiccating microclimate than the uninhabited hives in the dry season, but a slightly more desiccating microclimate in the wet. These patterns obviously result from a greater introduction of water vapour (absolute humidity) in the inhabited hives during the dry season, but a reduction of water vapour in the wet season, compared with the uninhabited hives. Regulation of hive humidity has been documented before for social insects (Bollazzi and Roces, 2010; Ellis et al., 2010; Human et al., 2006). For example, honeybees, and some species of ants and wasps maintain a high and stable nest RH (Bollazzi and Roces, 2010; Ellis et al., 2010; Human et al., 2006; Weidenmüller et al., 2002). A mechanism for regulating a higher-than-ambient hive humidity is relatively straightforward as an inhabited hive should accumulate a higher RH than the environment because of retention of respiratory and cutaneous water lost by the inhabitants (Woods and Smith, 2010) and water can be introduced into a hive through a number of avenues such as bees carrying in nectar and water (Cauich et al., 2004; Kühnholz and Seeley, 1997; Lindauer, 1955a). Furthermore, given the small entrance to the hive relative to its volume (Fig. 1), the opportunity for diffusion of water out of the hive is quite low. Alternatively, in an adaptation that appears to be unique, *A. essingtoni* appear able to maintain the RH inside their hives at lower levels than the ambient conditions during the summer wet season (Fig. 4). While advantages such as reduced likelihood of fungal infection can be readily hypothesised, the mechanism for this is less obvious. We speculate that latrines with high levels of uric acid may be hygroscopic (Werner, 1937), analogous to chemical desiccants, and that waste removal would remove water from the hive. Future studies could readily test these speculations by challenging the bees with experimental humidification or desiccation of the hive microclimate and monitoring the RH and the content of hive latrines.

Previous studies of hive humidity regulation by honeybees and bumblebees have shown that humidity optima may vary in different locations of the nest. For example, several studies have found that humidity is higher in the brood area compared with the nest cavity and nectar stores, although the mechanism continues to attract

discussion (Ellis et al., 2010; Human et al., 2006; Weidenmüller et al., 2002). We measured the atmosphere of the main chamber of the hive of *A. essingtoni*. Their hives are less spatially organised than those of other species studied previously, so we cannot speculate as to how effectively different areas of their hive may be RH regulated. It would be interesting for future studies to understand where and how hive humidity is regulated in various parts of the hive structure, including the brood, for Australian stingless bees and the underlying physiological or ecological imperatives for such controls, such as increased development rate or fecundity. Furthermore, given that there are differences in mass and productivity between our hives (Table S1), these may relate to the location of the hives in the landscape, and their capacity to regulate their hives to a physiological optimum. Studies of the selection of nest sites by wild hives would provide further insight into the role of behavioural regulation of the hive, as well as physiological regulation.

Conclusions

Although *A. essingtoni* show seasonal acclimatisation in EWL, thermal tolerance and $\dot{V}_{CO_2}$, only the direction of correlation between EWL and climatic conditions is consistent with our expectations of lower EWL in more desiccating conditions. The acclimatisation of EWL suggests that perhaps adaptations that promote water balance are more important for *A. essingtoni* than the thermal environment, in the arid tropics. The lesser importance of adaptations to the thermal environment is supported by the unexpected acclimatisation pattern for $\dot{V}_{CO_2}$ and thermal tolerance and by the lack of hive thermoregulation. Instead, *A. essingtoni* regulate the humidity of hives independent of ambient conditions during the dry winter, increasing the amount of water vapour in the hive. This creates a less desiccating microclimate within the hive and may provide a refuge for adult bees from the challenges of their environment in the dry winter. Water conservation may be linked with increased thermal tolerances, because higher thermal tolerance occurs in the cooler dry winter in these bees. Although it is possible that dehydration of bees in the wet season resulted in metabolic inhibition at lower temperatures than in the dry season, the mechanism linking thermal tolerance to the EWL patterns that we observed remains to be investigated.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.A. undertook all data collection, initial analysis and initial manuscript preparation in contribution to a BSc (Hons) degree at the University of Western Australia. S.T. and R.D.P. provided on-site supervision in collaboration with K.W.D. and P.C.W. Subsequent re-analysis of the data was undertaken by S.T., in consultation with R.D.P. and P.C.W. Interpretation and manuscript preparation was a joint exercise by S.T., R.D.P., K.W.D. and P.C.W.

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Supplementary information

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